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13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this project was to elucidate the long-term multigenerational consequences for a population of fish inhabiting a polluted estuary. The project studies in detail a population of killifish (Fundulus heteroclitus) inhabiting a portion of the Elizabeth River, VA that is highly polluted with polycyclic aromatic hydrocarbons (PAHs) and related chemicals that emanated from a former wood treatment facility that used creosote. Key results included: (1) This population has adapted to this pollution. (2) However, fitness costs, such as increased sensitivity to hypoxia, were incurred. (3) These adaptations and fitness costs are in part genetically-based. (4) The molecular bases for these adaptations include alterations in the aryl hydrocarbon receptor signaling pathway and in antioxidant defense systems. (5) The dominant toxicity produced by PAH mixtures in killifish is perturbed cardiovascular development in embryos. (6) Different PAHs with different modes of action were highly synergistic in the context of developmental toxicity. This synergy has important implications for environmental assessments of PAHs.					
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Adaptation of a Population of *Fundulus heteroclitus* to a Creosote-contaminated Environment: Mechanisms, Genetic Consequences, and Fitness Trade-offs.

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LONG-TERM GOALS

The long-term goal of this project is to understand the genetically- and physiologically-based processes by which a population of killifish (*Fundulus heteroclitus*) inhabiting the Elizabeth River, Virginia has adapted to its highly polluted environment, and to determine if fitness trade-offs are associated with these adaptations.

OBJECTIVES

The specific objectives of this project are: (1) to identify the contaminant(s) to which the Atlantic Wood site population of Elizabeth River *Fundulus* has adapted; (2) to elucidate biochemical mechanisms of adaptation in this population of *Fundulus*; (3) to elucidate selected genetic aspects of adaptation in this population of *Fundulus*; (4) to develop useful markers for adaptation and genetic change associated with multi-generation exposures in an estuarine model (*Fundulus*); and (5) to elucidate "costs" of adaptation associated with multi-generation exposures, based upon studies of Elizabeth River *Fundulus*.

APPROACH

For many aspects of this project, wild-caught killifish from the contaminated site (Atlantic Wood site, Elizabeth River, VA) and from a clean reference site (King's Creek, near the York River, VA) were bred in the laboratory for several generations. By comparing contaminated site versus reference site fish, along with comparisons among generations, we have elucidated differences in these populations and determine to what extent observed differences are genetically-based. Additionally, we examined fish biochemistry and molecular biology directly upon capture in the field. Doctoral candidates Joel Meyer and Deena Wassenberg were the key investigators on most aspects of this project. Additionally, doctoral student Alicia Timme and Master's student Lisa Bakansas were also involved in latter stages of the project. Additional assistance was provided by Laboratory Manager Lee Barber.

SUMMARY OF WORK COMPLETED

In Year 1, the Phase 1 Toxicity Identification and Evaluation (TIE) portion of the chemical identification studies, as well as toxicity screens for several model compounds were completed. A large portion of the studies elucidating cellular differences between ER and KC populations of

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killifish, the inheritability of these differences, and their associations with adaptation to Elizabeth River contaminants were completed. Studies designed to address the issue of fitness costs were also completed.

The Phase 1 TIE studies indicated that toxicants occurring in Elizabeth River likely responsible for driving adaptation in resident killifish are non-polar organics (versus metals, polar organics, or direct oxidants). Given the importance of creosote in the contamination of the study site, this is not surprising. However, creosote is a complex mixture of hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs), N-substituted hydrocarbons (such as carbazoles and acridines), and S-substituted hydrocarbons (such as thiophenes). See Yang (2001) for details.

The studies of cellular adaptations yielded several key findings. First, we wanted to confirm reports of adaptation (i.e., increased survival by ER killifish versus KC killifish exposed to ER sediment or sediment pore water) and determine whether any observed resistance by ER killifish was inheritable. As shown in Figure 1, ER F1's (i.e., offspring of field-collected adults) were significantly more resistant to ER sediment pore water than KC F1's. However, resistance exhibited by ER F2's was intermediate between ER F1's and KC F1's. These results suggest that adaptation by ER killifish is partially, but not entirely, genetically based. Variants of this experiment have been repeated many times with similar results. See Meyer and Di Giulio (2002, 2003) for details.

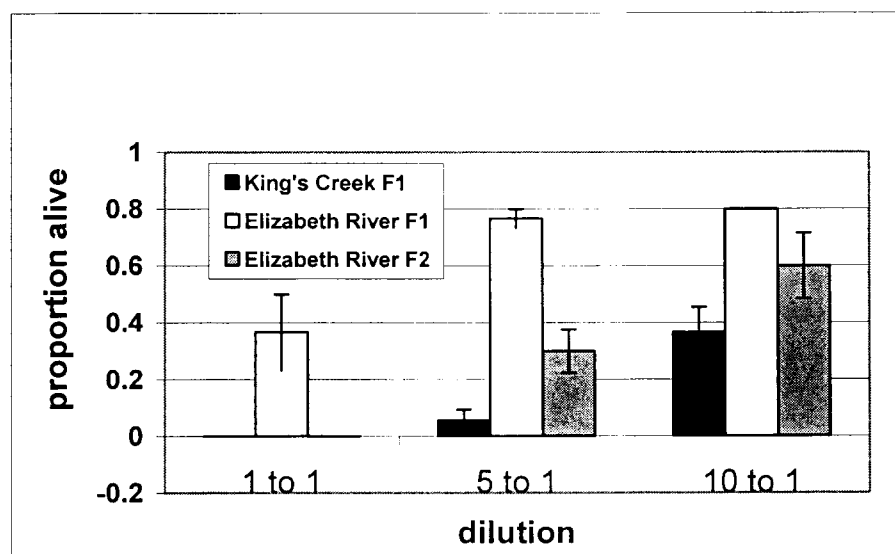


Figure 1. Larval survival at 14 days in ER sediment pore water dilutions. Dilutions are clean seawater to ER pore water ratios.

Other investigators have reported a down regulation of CYP1A expression, which is regulated through the AhR, in killifish from other sites that appear adapted to coplanar AhR agonists such as certain PCBs and dioxins. That is, killifish inhabiting systems highly contaminated with chemicals that normally induce CYP 1A are recalcitrant to CYP1A induction, both in the field and in the laboratory

following exposures to model inducers. Moreover, this down regulation has been associated with the chemical resistance concomitantly displayed by these fish.

Because some of the PAHs present in ER sediments are AhR agonists and previous studies have shown recalcitrance to CYP1A induction by adult killifish collected from the ER, we investigated this phenomenon in detail. To summarize this work, we consistently observed two key findings: (1) While ER F1 embryos display a pronounced lack of CYP1A inducibility (both with ER sediment pore water and potent model inducers such as BNF and 3MC), this effect is not observed in ER F2's (see Figure 2). That is, this phenomenon does not appear to be genetically based in ER killifish. (2) This lack of inducibility does not appear to be associated with increased resistance to ER sediment pore water. See Meyer and Di Giulio (2002) and Meyer et al. (2002) for details. The latter, surprising finding could be discovered by virtue of the fact that the *in ovo* EROD assay is non-destructive. That is, embryos can be assayed for EROD activity *in vivo*, then hatched and the resulting larvae employed for other studies, such as toxicity tests with pore waters as reported here. This technique, developed in part in conjunction with this project is described in detail in Nacci et al. (2005).

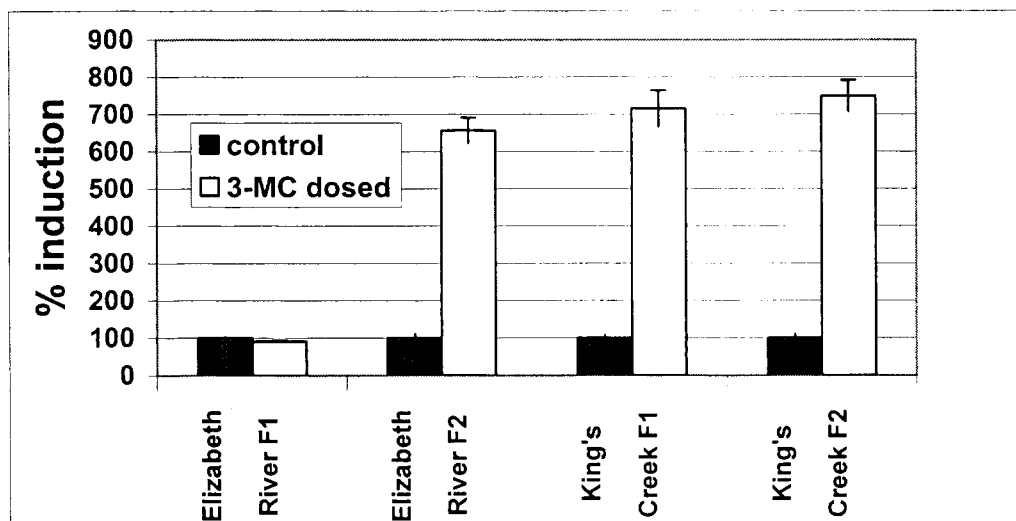


Figure 2. *CYP1A* response as measured by EROD activity after *in ovo* exposures of killifish embryos to 3-methylcholanthrene.

We also addressed in some detail the hypothesis that adaptation to their contaminated environment exacts fitness costs from ER killifish – in adapting to this environment, resident fish become less fit to deal with other, including natural, environmental stressors. Results supported this hypothesis. Relative to KC fish, ER progeny are more sensitive to UV-mediated phototoxicity with model phototoxic PAHs (anthracene and fluoranthene), to hypoxia, and they display lower rates of survival and growth under normal, clean water laboratory conditions. Hypoxia comprises a potentially important natural stressor that these estuarine organisms are likely to face. As shown in Figure 3, both ER F1s and F2s are far more sensitive to reductions in dissolved oxygen than KC F1s. These studies are described in Meyer and Di Giulio (2003).

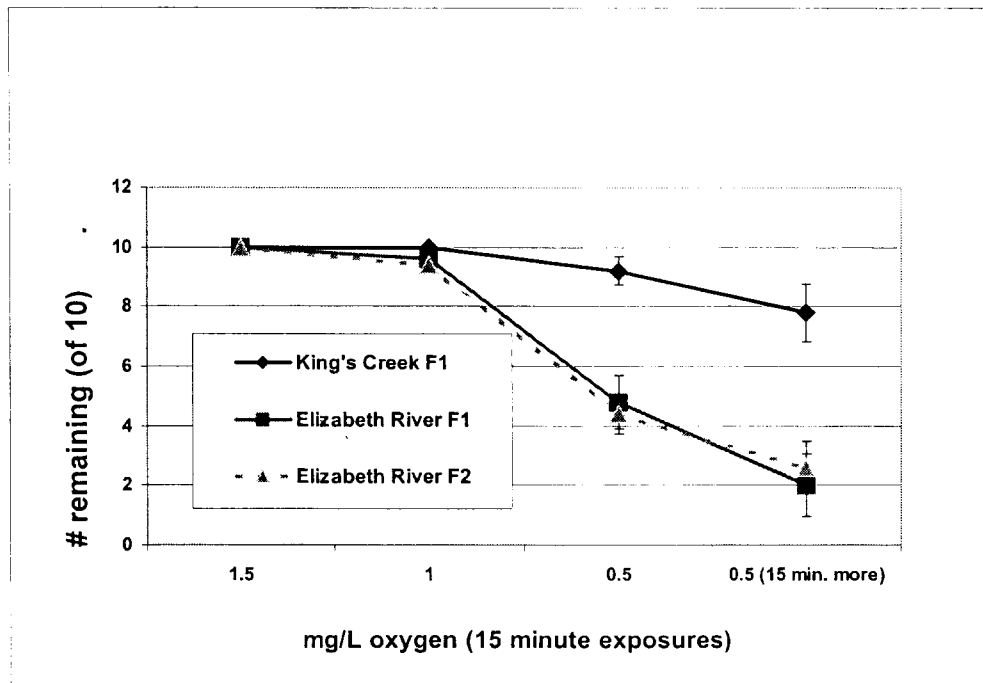


Figure 3. Larval tolerance to low oxygen conditions. # remaining – fish responding to stimuli after designated dissolved oxygen condition.

Collectively, experiments testing the hypothesis that ER killifish are more resistant to oxidative stress and that this adaptation is heritable have supported that hypothesis. For example, both ER F1's and ER F2's are more resistant to the acute toxicity of the model oxidant tert-butyl peroxide. Additionally, exposure to Elizabeth River sediments results in marked upregulation of many antioxidant defenses (e.g. total hepatic glutathione levels, as shown in Figure 4), but only a few are apparently genetically different in the Elizabeth River killifish (e.g. manganese superoxide dismutase, as shown in Figure 5). In accordance with this result, experiments addressing biochemical aspects of resistance in ER killifish have indicated generally elevated levels of antioxidants. Particularly, concentrations of GSH and TOSC appear to be elevated in ER larvae versus KC larvae. Also, elevated expression of SOD has been observed in ER F1's challenged with ER pore water versus KC F1's, although basal expression was similar. However, no clear patterns have been observed with enzymes involved in GSH synthesis and utilization. See Meyer et al. (2003) for details of these oxidative stress laboratory studies. In a field study, we also observed indications of elevated oxidative stress in ER fish versus KC fish (Bacanskas et al., 2004).

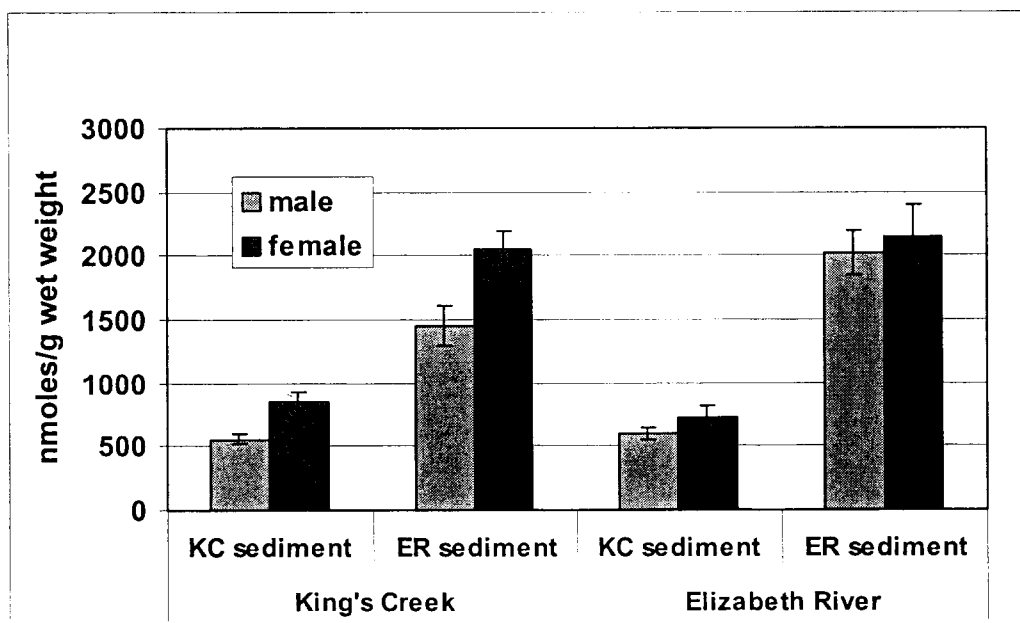


Figure 4: Total glutathione in adult livers (Elizabeth River F2 and King's Creek F1) is elevated after exposure to Elizabeth River sediments

Figure 4 – Explanatory Note: Some of the contaminants present at the Elizabeth River site can exert toxicity by causing oxidative stress. We have analyzed the levels of various antioxidant enzymes and chemicals in Elizabeth River and reference site (King's Creek) killifish, and found that many defenses are upregulated in response to exposure to Elizabeth River sediments (apparently adaptive responses). Shown in Figure 2 are levels of hepatic glutathione (an important low molecular weight antioxidant) in Elizabeth River and King's Creek killifish after exposure to either Elizabeth River or King's Creek (clean) sediments. Both populations of killifish produced much more glutathione in the presence of Elizabeth River contaminants; however, this response does not appear to be a genetically-based defense in Elizabeth River killifish.

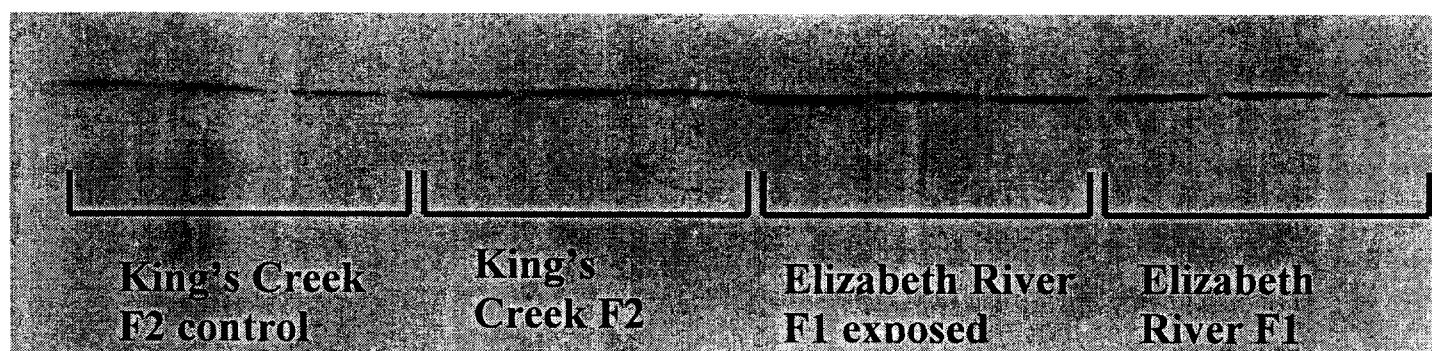
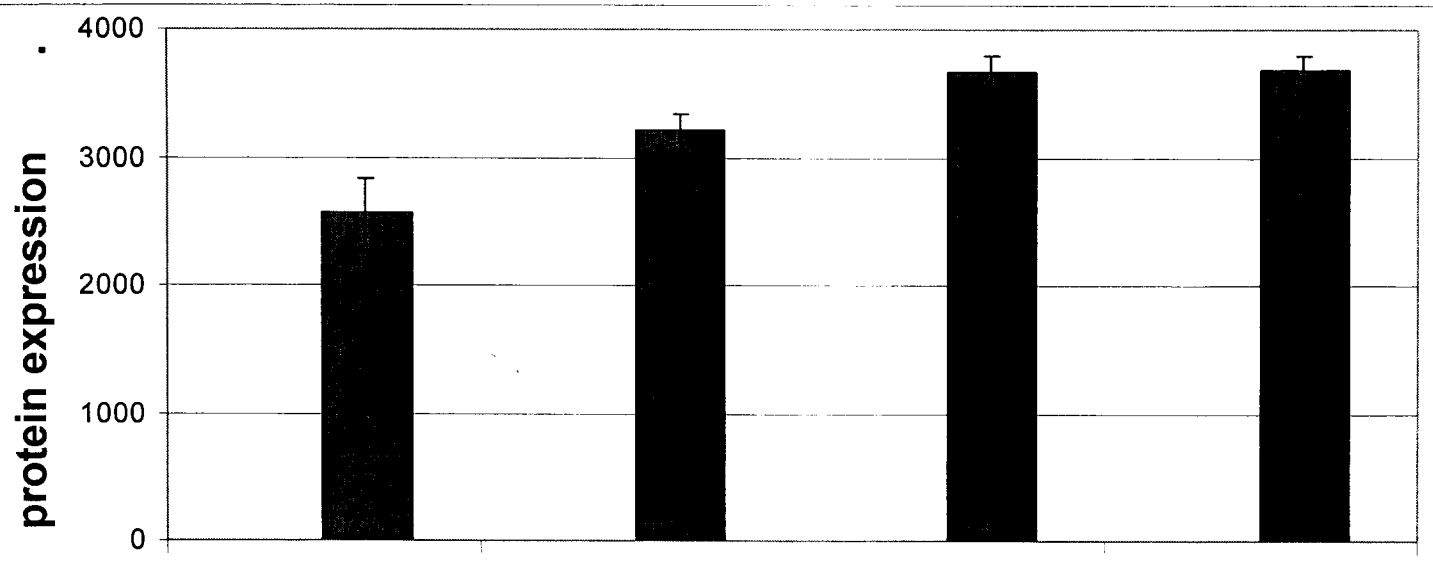


Figure 5: Mn SOD protein expression is elevated constitutively, but not induced, in Elizabeth River sediment-exposed larvae (whole body homogenates).

Figure 5 – Explanatory Note: Some antioxidant defenses appear to be upregulated permanently (genetically) in Elizabeth River killifish, and manganese superoxide dismutase (MnSOD; Figure 3) is one example. MnSOD is the mitochondrial form of this important antioxidant enzyme. In reference site (King's Creek) killifish, initially lower levels of MnSOD expression approach the constitutive levels of expression in Elizabeth River killifish after exposure to Elizabeth River sediments.

Following our observations described above concerning the lack of inducibility of CYP1A in ER killifish, we explored mechanisms underlying this recalcitrant phenotype. In collaboration with Dr. Mark Hahn and Dr. Sibel Karchner of Woods Hole Oceanographic Institution, we compared mRNA expression levels of genes involved in the aryl hydrocarbon receptor (AhR) pathway via semi-quantitative RT-PCR. These studies established that the altered expression of CYP1A is not due to marked alterations in expression of AhR pathway genes (e.g., the AhR repressor, shown in Figure 6, as well as AHR and ARNT). These studies are described in Meyer et al. (2003). We next tested the

Additionally, 22 sex-specific differences were observed. Among those genes differentially expressed between ER and KC fish were UDPglucose pyrophosphorylase (lower in ER fish) and glucose 6-phosphatase (higher in ER fish). These results are consistent with a greater reliance by ER fish on glycolytic metabolism, and are consistent with other observations concerning mitochondrial dysfunction and greater sensitivity to hypoxia in these fish. These studies are described in Meyer et al. (2005).

In earlier stages of this project, we established that a key toxicity in killifish embryos exposed to extracts of Elizabeth River sediments were cardiovascular deformities including pericardial edema and "tube heart" (Meyer et al., 2002). This was among the effects that killifish from the Elizabeth River site were resistant to, relative to fish from reference sites (York River, VA and Beaufort, NC). In the final stage of this project, we explored mechanisms by which these extracts were teratogenic, a phenomenon complicated by the complex mixture of chemicals they contain. We performed a dose-response analysis, examining dose response curves for different dilutions of sediment extract and CYP1A activity (measured as ethoxyresorufin-O-deethylase, EROD), and between the dilutions and a quantitative score for severity of cardiovascular deformities. Results (representative ones illustrated in Figure 7) revealed a positive relationship between dose (lower dilutions corresponding to higher dose) and EROD activities, until a peak is reached (at about a 1:50 dilution). Thereafter, EROD activities decline with higher dose. Deformities are not observed at doses corresponding to increasing or peak EROD activities. However, as EROD substantially declines, at 1:10 and 1:5 dilutions, deformities arise and demonstrate dose-response (Figure 7). These studies are described in Wassenberg and Di Giulio (2004b).

These curves led us to explore interactions between CYP1A inducers and CYP1A inhibitors in connection to cardiovascular teratogenesis. Our hypothesis was that, consistent with the literature and our previous work, is that at lower concentrations, CYP1A is induced, that reaches a peak, after which it declines due to elevated production of reactive oxygen species (ROS); this ROS production can inhibit CYP1A activity and cause developmental perturbations. This hypothesis was supported by studies with model compounds for this extract. For example, we have investigated interactions between model polycyclic aromatic hydrocarbon (PAH) type inducers and inhibitors, β -naphthoflavone (BNF) and α -naphthoflavone (ANF), respectively. A similar trend as observed with the sediment extract is observed (Figure 8). ANF by itself neither induces EROD (as expected) nor causes deformities. BNF induces EROD, but at the concentrations employed, has no effect on development. However, in a dose-dependent fashion, ANF concomitantly inhibits BNF-induced EROD activity, and deformities arise as this inhibition occurs (Figure 8). ANF is known to be both a CYP1A enzyme inhibitor and an aryl hydrocarbon receptor (AHR) antagonist. Importantly, these experiments, described in Wassenberg and Di Giulio (2004a) demonstrate a very potent synergy between PAHs that act as AHR agonists and other PAHs that act as CYP1A inhibitors. Similar results were observed when binary combinations of PAHs occurring in natural systems such as the ER were employed. For those studies, we employed benzo[a]pyrene as the AHR agonist and either carbazole or dibenzothiophene as the CYP1A inhibitor (Wassenberg et al in press). Current risk assessments for environmental PAH mixtures employ additive models of toxicity. Thus, our findings of synergistic toxicity may have important ramifications for PAH risk assessment.

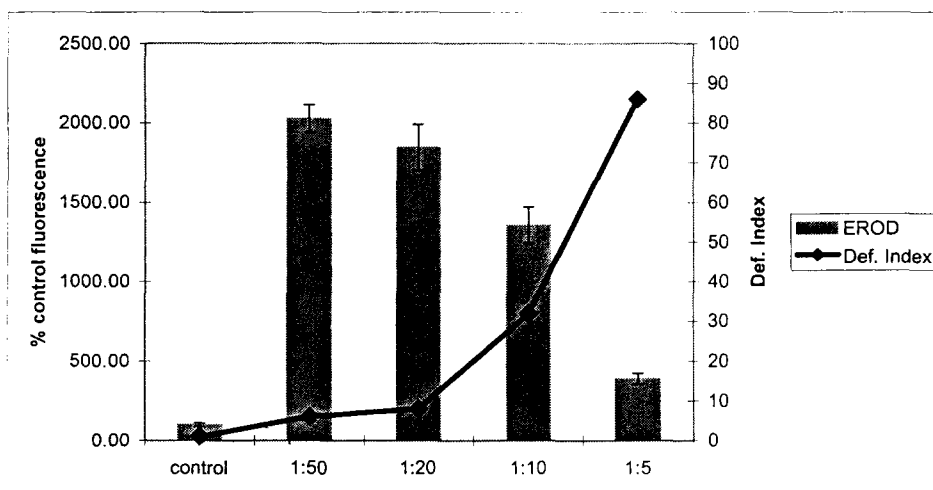


Figure 7. Effect of reducing dilution of Elizabeth River sediment extract (i.e., increasing contaminant concentration) on EROD activities and cardiovascular deformities in *Fundulus heteroclitus* embryos.

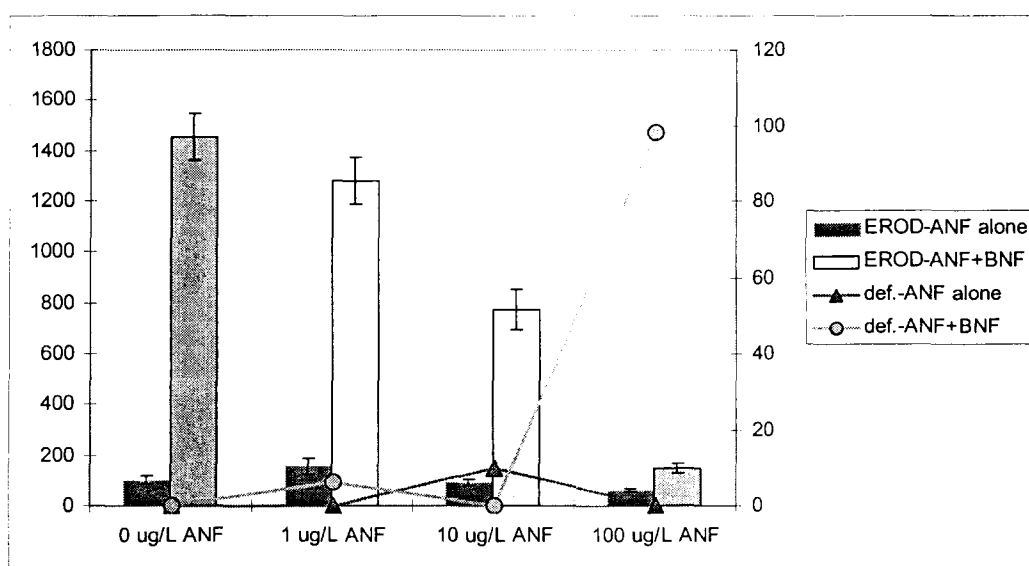


Figure 8. Effect of increasing concentrations of ANF on EROD activities and cardiovascular deformities in *Fundulus heteroclitus* embryos concomitantly exposed to BNF.

IMPACT/APPLICATIONS

This work was directed at understanding at the biochemical and molecular levels how organisms such as fish are able to evolve genetically in response to high levels of contaminants, and whether there are costs (fitness trade-offs) associated with the process of adaptation. Additionally, we elucidated

mechanisms by which a real world chemical mixture causes cardiovascular teratogenesis. The results represent important information on the long-term, multigenerational effects of chemical contamination, which will be useful to regulators and risk assessors who must determine what levels of chemicals should be allowed in the environment. Our discovery of synergistic PAH toxicity in developing embryos also has important regulatory implications. In addition, an understanding of these effects and the mechanisms associated with them will provide tools for industry and government agencies (including the Department of Defense) which are involved in pollution monitoring, control, and clean-up, as well as evaluation of remediation efforts.

TRANSITIONS

Our information is being used by investigators studying related phenomena at the U.S. EPA and other institutions including Virginia Institute of Marine Science, and Woods Hole Oceanographic Institute (WHOI). We continue to collaborate with Dr. Mark Hahn's laboratory at WHOI on studies addressing mechanisms of PAH-mediated teratogenesis, and interactions between PAHs and hypoxia. This research, which emanated from this ONR project, is supported by NIEHS, through Duke's Superfund Basic Research Center (Richard Di Giulio, PI and Center Director).

RELATED PROJECTS

Scientists (including Drs. Michael Newman and Peter Van Veld) at the Virginia Institute of Marine Science have investigated related phenomena associated with multi-generational exposures in the ER, largely from a more population/community level perspective. Dr. Diane Nacci (U.S. EPA, Narragansett) continues to explore AHR-related mechanisms of adaptation in killifish from a PCB-contaminated site (New Bedford Harbor). Dr. Mark Hahn (Woods Hole) is investigating mechanisms underlying AHR down regulation in killifish from PCB and dioxin-contaminated environments, and interactions with hypoxia.

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